Transcriptional responses of *Anopheles gambiae* s.s mosquito larvae to chronic exposure of cadmium heavy metal
[version 2; peer review: 1 approved, 1 approved with reservations, 1 not approved]

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Abstract

Background: *Anopheles gambiae* larvae traditionally thrive in non-polluted environments. We previously documented the presence of the larvae in heavy metal polluted urban aquatic environments and the associated biological cost. The goal of this study was to unravel the molecular dynamics involved in the adaptation of the mosquitoes to the heavy metals.

Methods: Total RNA was extracted from third instar larvae of both cadmium treated populations and untreated control populations. The RNA concentrations were normalized and complementary DNAs were prepared. Then annealing control primer (ACP) technology was applied to establish transcriptional responses in *An. gambiae* larvae following several generational (n=90) chronic exposures to cadmium. Differentially expressed genes were determined by their differential banding patterns on an agarose gel. Gel extraction and purification was then carried out on the DEGs and these were later cloned and sequenced to establish the specific transcripts.

Results: We identified 14 differentially expressed transcripts in response to the cadmium exposure in the larvae. Most (11) of the transcripts were up-regulated in response to the cadmium exposure and were putatively functionally associated with metabolism, transport and protein synthesis processes. The transcripts included ATP-binding cassette transporter, eupolytin, ribosomal RNA,
transcription initiation factor, THO complex, lysosomal alpha-mannosidase, sodium-independent sulfate anion transporter and myotubularin related protein 2. The down-regulated transcripts were functionally associated with signal transduction and proteolytic activity and included Protein G12, adenylate cyclase and endoplasmic reticulum metallopeptidase.

**Conclusions:** Our findings shed light on pathways functionally associated with the adaptation to heavy metals that can be targeted in integrated vector control programs, and potential *An. gambiae* larvae biomarkers for assessment of environmental stress or contamination.

**Keywords**
Anopheles gambiae larvae, differentially expressed genes, cadmium, heavy metal tolerance

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**Corresponding author:** Catherine N. Muturi (katengambi@gmail.com)

**Author roles:** Muturi CN: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; Rono MK: Conceptualization, Writing – Review & Editing; Masiga DK: Conceptualization, Resources, Supervision, Writing – Review & Editing; Wachira FN: Supervision, Writing – Review & Editing; Ochieng R: Methodology, Resources; Mireji PO: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed.

**Grant information:** Funding for this study was provided by the Department of Research and Extension, Egerton University and the DAAD in-country Scholarship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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**How to cite this article:** Muturi CN, Rono MK, Masiga DK *et al.* Transcriptional responses of *Anopheles gambiae s.s* mosquito larvae to chronic exposure of cadmium heavy metal [version 2; peer review: 1 approved, 1 approved with reservations, 1 not approved] F1000Research 2018, 6:2173 https://doi.org/10.12688/f1000research.13062.2

**First published:** 22 Dec 2017, 6:2173 https://doi.org/10.12688/f1000research.13062.1
Methods

Sample insects

*Anopheles gambiae s.s* mosquitoes that had been collected from the Mbita field station (00.025’S, 34.013’E), Homa Bay County in Kenya were used for the study. The colony was kept in the Animal Rearing and Quarantine Unit (ARQU) at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. Larval stages of *Anopheles gambiae s.s.* were selected for tolerance to cadmium heavy metal through chronic exposures of Maximum Acceptable Toxicant Concentration (MATC) that had been empirically determined (Mireji et al., 2010a). Cadmium metal tolerant strains and control (untreated) strains of the mosquito were raised separately and in triplicates. All subsequent generations of the mosquito were subjected to chronic exposures of cadmium metal as described in Mireji et al., (2010a). Standard Operating Procedure (SOP) for the rearing of *Anopheles* mosquitoes was followed for colony maintenance (Ford & Green, 1972). Cadmium used in our study was applied as Cadmium Chloride (CdCl₂) 99.99% pure (Fisher Scientific LLC, Fair Lawn, NJ, U.S.A).

RNA isolation

Total RNA was extracted from the third instar larvae of experimental and control *An. gambiae* populations using Trizol® (Invitrogen). Quantification of the extracted RNA was done using the micro-spectrophotometer Genequant pro (Amersham Pharmacia Ltd., Bucks, UK) (Table 1) and a few samples were quantified using the NanoDrop® (Therm Scientific) (Table 2). In addition, DNaseI digestion was carried out to remove any residual DNA that could present in the extracted RNA. Total RNA that was isolated was stored at -80°C.

GeneFishing™ Reverse Transcription

The total RNA extracted from experimental and control *An. gambiae* populations were normalized to same concentrations and directly used for the synthesis of first strand complementary DNA (cDNA) using reverse transcriptase (Hwang et al., 2003). Reverse transcription was carried out in a final reaction volume of 20µl containing 2µg of the purified mRNA at 42°C for 1.5 hours. The components of the reaction were: 4µl of 5X reverse transcriptase (200U/µl, Promega). The synthesized cDNA digestion was carried out to remove any residual DNA that could present in the extracted RNA. Total RNA that was isolated was stored at -80°C awaiting PCR procedure.

ACP based- GeneFishing™ PCR

Annealing control primer based PCR using the GeneFishing™ DEG kit from Seegene, Seoul, South Korea (Kim et al., 2004), was used to determine differentially expressed genes in the heavy metal treated group and the control population.

Synthesis of the second strand cDNA and PCR was carried out in a single tube. The second strand was synthesized in one cycle of first stage PCR at 50°C, in a final reaction volume of 20µl. The components in the reaction tubes included 3–5µl of diluted first
Table 1. RNA Quantification of the Control samples (CT) and Cadmium treated samples (CD) that were extracted using the Trizol reagent (Invitrogen). RNA Quantification was done using the Micro-Spectrometer GeneQuant pro (Amersham Pharmacia Ltd, Bucks, UK).

<table>
<thead>
<tr>
<th>SAMPLE ID</th>
<th>A260/A280</th>
<th>RNA Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT10</td>
<td>1.887</td>
<td>236.04</td>
</tr>
<tr>
<td>CT11</td>
<td>1.840</td>
<td>180.96</td>
</tr>
<tr>
<td>CT12</td>
<td>1.858</td>
<td>153.52</td>
</tr>
<tr>
<td>CT13</td>
<td>1.899</td>
<td>264.65</td>
</tr>
<tr>
<td>CT14</td>
<td>1.850</td>
<td>189.21</td>
</tr>
<tr>
<td>CT15</td>
<td>1.899</td>
<td>241.36</td>
</tr>
<tr>
<td>CT16</td>
<td>1.865</td>
<td>230.27</td>
</tr>
<tr>
<td>CT17</td>
<td>1.843</td>
<td>158.25</td>
</tr>
<tr>
<td>CT18</td>
<td>1.881</td>
<td>188.18</td>
</tr>
<tr>
<td>CT19</td>
<td>1.869</td>
<td>190.38</td>
</tr>
<tr>
<td>CD10</td>
<td>1.930</td>
<td>296.83</td>
</tr>
<tr>
<td>CD11</td>
<td>1.898</td>
<td>240.82</td>
</tr>
<tr>
<td>CD12</td>
<td>1.913</td>
<td>236.82</td>
</tr>
<tr>
<td>CD13</td>
<td>1.933</td>
<td>225.05</td>
</tr>
<tr>
<td>CD14</td>
<td>1.934</td>
<td>283.98</td>
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<tr>
<td>CD15</td>
<td>1.853</td>
<td>187.16</td>
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<tr>
<td>CD16</td>
<td>1.845</td>
<td>244.09</td>
</tr>
<tr>
<td>CD17</td>
<td>1.918</td>
<td>252.15</td>
</tr>
<tr>
<td>CD18</td>
<td>1.923</td>
<td>298.63</td>
</tr>
<tr>
<td>CD19</td>
<td>1.898</td>
<td>153.81</td>
</tr>
</tbody>
</table>

Table 2. RNA Quantification of a few Control samples (CT) and Cadmium treated RNA samples (CD) that were extracted using the Trizol reagent (Invitrogen). Quantification was done using the NanoDrop® (Thermo Scientific).

<table>
<thead>
<tr>
<th>SAMPLE ID</th>
<th>RNA Concentration</th>
<th>UNIT</th>
<th>A260/A280</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT1</td>
<td>2115.2</td>
<td>ng/µl</td>
<td>1.96</td>
</tr>
<tr>
<td>CT2</td>
<td>3189.2</td>
<td>ng/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>CD1</td>
<td>1824.2</td>
<td>ng/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>CD2</td>
<td>3061.2</td>
<td>ng/µl</td>
<td>2.0</td>
</tr>
</tbody>
</table>

strand cDNA, 1µl 10Mm dT-ACP2 reverse primer (5’-CTGTG AATGCTGCGACTACGATIITIII(T)3’), 10µl 2x master mix (Seegene, Seoul, South Korea) and 1µl 10µM arbitrary ACP (forward primer).

PCR procedures for the synthesis of the second strand were completed in one cycle, at 94°C for 1 min then 50°C for 3min and 72°C for 1 min.

The second stage of the PCR protocol consisted of 40 cycles at 94°C for 40s, 65°C for 40s, 72°C for 40s and the final extension for 10 min at 72°C. 2% agarose gel electrophoresis with ethidium bromide staining was used for separation of the PCR products.

Gel extraction
Differentially expressed bands in the control and cadmium exposed population subjected to the same primer set were excised from the agarose gel using a scalpel under Ultra Violet illumination. The gel slices were then purified using the QIAquick® gel extraction kit (QIAGEN, Inc., Valencia, CA), following the instructions from the manufacturer.

Cloning
Gel-purified PCR products were directly cloned into a pGEMT Easy vector (Invitrogen, Carlsbad, CA, USA), using JM109 competent cells. Colonies were grown at 37°C for 18 hours on Luria broth agar plates, containing ampicillin, X-gal and IPTG for blue/white colony screening. Cloned plasmids were then purified using the GeneJET™ Miniprep kit (Fermentus, Thermo Fisher Scientific Inc.), as per the instructions from the manufacturer.

Sequencing
Sequencing was done with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using M13 primers. The sequences were edited using VecScreen and BioEdit software. Edited sequences were analyzed by searching for similarities in VectorBase against the Anopheles gambiae PEST strain transcripts sequences, AgamP4.6 geneset using the BLASTn search program (Altschul et al., 1990).

Results
We successfully implemented the ACP system to identify differentially expressed genes (DEGs) in larvae chronically exposed to cadmium, as previously demonstrated in blastocyst experiments (Cui et al., 2005; Hwang et al., 2004; Hwang et al., 2005). Our differential banding patterns of the cDNA representation of DEGs is summarized in Figure 1. Fourteen DEGs were identified after chronic exposure of An. gambiae larvae to cadmium heavy metal (Table 3). Most (11) of the differentially expressed genes were induced in cadmium exposed relative to the cadmium un-exposed controls. Our BLAST (REF) results revealed that the cadmium induced transcripts were clustered into metabolism (AGAP008584-RA, AGAP001249-RA and AGAP009563-RA), transport (AGAP012302-RA and AGAP002638-RA) and protein synthesis (AGAP028915-RA, AGAP004750-RA, AGAP028391-RA, AGAP003870-RA, AGAP028907-RA, AGAP028818-RA and AGAP028899-RA) processes.

Three of the DEGs identified were suppressed in the cadmium exposed larvae and these included AGAP006187-RA, AGAP002262-RA and AGAP003078-RA.

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Dataset 1. Sequence data obtained after sequence analysis using the BioEdit software

http://dx.doi.org/10.5256/f1000research.13062.d18704

The sequences were subsequently taken through a BLAST search. The results of the sequence analysis are shown on the manuscript.
Figure 1. Differential cDNA banding patterns in cadmium treated and control population of mosquito larvae. The arrows indicate the DEGs observed using ACP 75, ACP 76 and ACP 78 primer set. Number 1 represents Cadmium population while 2 represents control population. M= 50bp molecular marker. High intensity of a band represents an up-regulation of a particular gene in cadmium or control population.

Table 3. Blastn results from VectorBase. Sequence data obtained was blasted against Anopheles gambiae PEST strain transcript sequences, AgamP4.6 geneset in May 2017.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>Description of gene product</th>
<th>E-Value</th>
<th>% ID</th>
<th>Expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGAP002638-RA</td>
<td>ABCH1</td>
<td>ATP-binding cassette transporter (ABC transporter) family H member 1</td>
<td>3</td>
<td>77.5</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP001249-RA</td>
<td>Eupolytin</td>
<td></td>
<td>3e-31</td>
<td>98.7</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP028915-RA</td>
<td>SSU_rRNA_eukaryotic</td>
<td>Eukaryotic small subunit ribosomal RNA</td>
<td>8e-79</td>
<td>98.2</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP004750-RA</td>
<td>Translation initiation factor 4G</td>
<td>6.4</td>
<td>87</td>
<td>Up</td>
<td></td>
</tr>
<tr>
<td>AGAP028915-RA</td>
<td>SSU_rRNA_eukaryotic</td>
<td>Eukaryotic small subunit ribosomal RNA</td>
<td>8e-78</td>
<td>99.4</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP006187-RA</td>
<td>Protein G12</td>
<td></td>
<td>6.8</td>
<td>100</td>
<td>Down</td>
</tr>
<tr>
<td>AGAP003078-RA</td>
<td>Endoplasmic reticulum metallopeptidase 1</td>
<td></td>
<td>1.5</td>
<td>80.6</td>
<td>Down</td>
</tr>
<tr>
<td>AGAP028391-RA</td>
<td>Isu rRNA</td>
<td></td>
<td>3e-103</td>
<td>100</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP028915-RA</td>
<td>SSU_rRNA_eukaryotic</td>
<td>Eukaryotic small subunit ribosomal RNA</td>
<td>4e-49</td>
<td>96.6</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP028915-RA</td>
<td>SSU_rRNA_eukaryotic</td>
<td>Eukaryotic small subunit ribosomal RNA</td>
<td>5e-81</td>
<td>98.8</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP003870-RA</td>
<td>Thoc7</td>
<td></td>
<td>6.4</td>
<td>87</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP008584-RA</td>
<td>Lysosomal alpha-mannosidase</td>
<td>3.4</td>
<td>90.5</td>
<td>Up</td>
<td></td>
</tr>
<tr>
<td>AGAP010252-RA</td>
<td>RplL23</td>
<td>60S ribosomal protein L23</td>
<td>4e-12</td>
<td>100</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP028907-RA</td>
<td>SSU_rRNA_eukaryotic</td>
<td>Eukaryotic small subunit ribosomal RNA</td>
<td>3e-06</td>
<td>91.2</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP028818-RA</td>
<td>5_8S_rRNA</td>
<td>5.8S ribosomal RNA</td>
<td>3e-37</td>
<td>98.9</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP028899-RA</td>
<td>SSU_rRNA_eukaryotic</td>
<td>Eukaryotic small subunit ribosomal RNA</td>
<td>2e-08</td>
<td>100</td>
<td>Up</td>
</tr>
<tr>
<td>AGAP009563-RA</td>
<td>Myotubularin related protein 2</td>
<td>0.74</td>
<td>91.3</td>
<td>Up</td>
<td></td>
</tr>
<tr>
<td>AGAP002262-RA</td>
<td>Adenylyl cyclase 8</td>
<td>9.6</td>
<td>100</td>
<td>Down</td>
<td></td>
</tr>
<tr>
<td>AGAP012302-RA</td>
<td>Sodium-independent sulfate anion transporter</td>
<td>0.36</td>
<td>88.9</td>
<td>Up</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

We identified ATP-binding cassette transporters belonging to the superfamily of membrane proteins that are present in all living organisms (Dean & Annilo, 2005). They are normally associated with movement of substrates such as amino acids, peptides, sugars, metals, inorganic ions, lipids, lipopolysaccharides and xenobiotics across biological membranes (Dawson & Locher, 2006; Hollenstein et al., 2007a). The ABC transporters have been shown to affect development, metabolism and insecticide resistance in insects (Borycz et al., 2008; Dow & Davies, 2006; Ricardo & Lehmann, 2009; Vache et al., 2007). The silencing of the ABCH1 gene has been shown to result in the death of larvae and pupae (Guo et al., 2015). Therefore, induction of the ABC transporters may suggest that they are involved in cadmium transport through membranes to reduce toxicity of cadmium metal to the larvae in their environment.

The induction of the eupolytin gene may have a role in the activation of defense molecules. In a study involving the ground beetle Eupolyphaga sinensis, eupolytin-1 gene encoding a protease was shown to be involved in the activation of plasminogen and hydrolysis of fibrinogen (Yang et al., 2011).

Ribosomal genes are involved in protein synthesis and upregulation of the various arrays of ribosomal RNAs in this study, which suggests their role in enhancing the survival of An. gambiae in the heavy metal polluted environment by the transcription and translation of genes which are important in the adaptation of the larvae to xenobiotics.

The nuclear structure referred to as THO complex is usually conserved in all kingdoms, and it has an important role in the packing of pre-mRNA molecules into RNA-protein assemblies which are termed mRNPs (Köhler & Hurt, 2007). A study carried out recently has shown that the THO complex is required for efficient expression of some genes, ensuring genetic stability thereby preventing transcription-associated recombination (Gewartowski et al., 2012). The expression of the THO complex is suggestive of its role in expressing defense genes that enhance survival of larvae in a Cadmium polluted environment.

Suppression of AGAP006187-RA, AGAP002262-RA and AGAP003078-RA transcripts that included G-Proteins couple receptors to adenyl cyclase stimulation indicated increasing levels of cAMP and a cascade of events that constitute the signal transduction pathway that drive cellular responses. Metalloproteinases are a ubiquitous and diverse group of enzymes containing both endopeptidases and exopeptidases. Although they vary widely at the sequence, structural, and functional levels, they all require a metal ion for catalytic activity (Rawlings & Salvesen, 2013). The suppression of these important genes involved in signal transduction and proteolytic activity would account for the larval mortality rates that are usually observed in larvae raised in the cadmium heavy metal environment.

Our findings shed light on the adaptation of the An. gambiae mosquito to heavy metals by differentially expressing particular genes in response to a toxicant impact. A study to determine differentially expressed genes in cadmium-exposed sebastes schlegeli unraveled genes related to pathogenesis, extrinsic stresses, and catalytic metabolites (Woo & Yum, 2014). Previous studies have indicated that metallothionein and α-tubulin genes that are present in insects can be used as potential biomarkers (Hare, 1992; Klerks & Weis, 1987; Mattingly et al., 2001; Roesijadi, 1994). Metallothionein was assessed through C. quinquefasciatus mosquito larvae for Copper, Cadmium and Zinc aquatic environmental levels (Sarkar et al., 2004). Therefore, the genes identified might be useful in the development of potential biomarkers that can be used to assess the level of environmental pollution of heavy metal origin in An. gambiae mosquitoes.

Conclusions

We were able to identify genes that are differentially expressed due to chronic exposure of An. gambiae larvae to cadmium metal using the ACP-based PCR method. However, application of more sensitive techniques like those used in proteomics would generate more data.

Data availability

Dataset 1: Sequence data obtained after sequence analysis using the BioEdit software. The sequences were subsequently taken through a BLAST search. The results of the sequence analysis are shown on the manuscript. DOI, 10.5256/f1000research.13062.d187045 (Muturi et al., 2017a).

Dataset 2: Sample of the colony PCR experiment. The gel photo of a colony PCR of 20 samples that was carried out after blue/white colony screening using M13 primers. DOI, 10.5256/f1000research.13062.d187046 (Muturi et al., 2017b).

Competing interests

No competing interests were disclosed.

Grant information

Funding for this study was provided by the Department of Research and Extension, Egerton University and the DAAD in-country Scholarship.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We hereby wish to acknowledge the following individuals for their contribution to this work:

The Head of the Capacity Building Department at ICIPE, for granting us permission to carry out this work in their Molecular and Biotechnology unit.

The Director of the Research and Extension Department at Egerton University.

The DAAD team for the financial support, which enabled this work to be completed.
Open Peer Review

Current Peer Review Status: ⚫️ ✓ ❌

Version 2

Reviewer Report 04 February 2019

https://doi.org/10.5256/f1000research.16709.r43256

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Dick Roelofs

Department of Ecological Science, Faculty of Science, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

The authors apply a differential PCR method to discover and identify differentially expresses genes between control A. gambiae populations and populations adapted to cadmium. 14 differentially expressed transcripts were identified and annotated upon sequencing.

While the RNA extraction and initial cDNA synthesis were executed properly, I'm worried about the follow up steps and the verification of up- or down regulation.

Major Issues:

- Second stage PCR of 40 cycles may introduce tremendous bias in fragments that differ in PCR efficiency. After so many cycles only shorter fragments will be preferentially amplified. This may in part explain why so few DEGs have been identified in this study. All other competing DEGs with lower efficiency will be lost.

- The GeneFishing technique is outdated. I would rather recommend SSH, microarray analysis or RNAseq. All of these techniques would give a much more complete picture of genetic differences in the Cd adapted populations.

- It is essential to verify DEGs using for instance QPCR. I used to work with differential PCR screening, and quite a number of the initial leads were false positives. So, an independent second method should be used to validate the DEGs

- Most of the identified DEGs are associated with rRNA indicating that the dT-ACP 1 primer was not specific enough to target mRNA. This is very worrying and confirms my 1st issue raised.

In conclusion, these are very preliminary data generated with an out dated system.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Ecological genomics, transcriptomics, comparative genomics. Molecular ecology of stress adaptation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 25 June 2018

https://doi.org/10.5256/f1000research.16709.r35426

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Shüné V. Oliver

Centre for Emerging, Zoonotic and Parasitic Diseases Centre for Emerging, Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases, Johannesburg, South Africa

I am satisfied with the requisite methodological changes.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly
Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

**Version 1**

Reviewer Report 29 May 2018

https://doi.org/10.5256/f1000research.14161.r34199

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Shüné V. Oliver

Centre for Emerging, Zoonotic and Parasitic Diseases Centre for Emerging, Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases, Johannesburg, South Africa

This manuscript is technically sound, and presents a worthwhile body of research. I would recommend accepting. However, there is one major correction that must be made before accepting. Please give details about the assessment of RNA integrity. Without this, the other results are not valid, so please detail about your confirmation of RNA integrity post extraction.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes
If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

---

Author Response 11 Jun 2018

**Catherine Ngambi**, Egerton University, Egerton, Kenya

Thanks so much for your positive review of the manuscript.

I am sorry, I had not given the data about the RNA integrity but had only stated that I had quantified the extracted RNA using the microspectrophotometer genequant pro.

I have revised the manuscript and have indicated the quantification results and the equipments used.

To estimate RNA purity, the ratio of the Absorbance contributed by the RNA to the Absorbance of the contaminants is calculated. The acceptable ratios for purity or typical requirements for A260/A280 ratios are 1.8-2.2.

The samples that were use for the downstream application were those that met the purity criteria of 1.8-2.2, that is the ratios for A260/A280. Therefore, the revised document now contains these details.

Kindest Regards,
Catherine.

**Competing Interests:** No competing interests.
David Essumang
Department of Chemistry, University of Cape Coast, Cape Coast, Ghana

The main work is outside my expertise and I was struggling to make some meaningful contribution. The bulk of the work is in molecular experimentation and I have limited knowledge in their methods. However, my major concern with the entomological aspect is that the authors did not show how long (the number of generations) the mosquitoes were exposed to the heavy metal during the selection. This would be helpful if someone wants to repeat the work.

Furthermore, the study did not show the impact of the biomarkers identified in the development, enzyme activities and insecticide resistance of the mosquitoes used for the study.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Environmental Scientist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
I wanted to respond to the question raised about the number of generations that mosquitoes were exposed to cadmium heavy metal. As stated in the Abstract its for 90 generations.

Kindest regards,
Catherine.

**Competing Interests:** No competing interests were disclosed.